



## UNIVERSITÀ DEGLI STUDI DI TORINO

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# The 5-HT<sub>7</sub> receptor triggers cerebellar long-term synaptic depression via PKC-MAPK

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## Abstract

The 5-HT<sub>7</sub> receptor (5-HT<sub>7</sub>R) mediates important physiological effects of serotonin, such as memory and emotion, and is emerging as a therapeutic target for the treatment of cognitive disorders and depression. Although previous studies have revealed an expression of 5-HT<sub>7</sub>R in cerebellum, particularly at Purkinje cells, its functional role and signaling mechanisms have never been described. Using patch-clamp recordings in cerebellar slices of adult mice, we investigated the effects of a selective 5-HT<sub>7</sub>R agonist, LP-211, on the main plastic site of the cerebellar cortex, the parallel fiber-Purkinje cell synapse. Here we show that 5-HT<sub>7</sub>R activation induces long-term depression of parallel fiber-Purkinje cell synapse via a postsynaptic mechanism that involves the PKC-MAPK signaling pathway. Moreover, a 5-HT<sub>7</sub>R antagonist abolished the expression of PF-LTD, produced by pairing parallel fiber stimulation with Purkinje cell depolarization; whereas, application of a 5-HT<sub>7</sub>R agonist impaired LTP induced by 1 Hz parallel fiber stimulation. Our results indicate for the first time that 5-HT<sub>7</sub>R exerts a fine regulation of cerebellar bidirectional synaptic plasticity that might be involved in cognitive processes and neuropsychiatric disorders involving the cerebellum.

**Keywords:** 5-HT<sub>7</sub> receptor; Cerebellum; Long-term depression; Purkinje cell; Serotonin; Synaptic transmission.

**Abbreviations:** 5-HT, Serotonin; 5-HT<sub>7</sub>R, Serotonin type 7 receptor; 5-HT<sub>1</sub>R, Serotonin type 1 receptor; 5-HT<sub>2</sub>R, Serotonin type 2 receptor; CF, climbing fiber; CF-EPSCs, excitatory postsynaptic currents evoked by climbing fiber stimulation; EPSCs, excitatory postsynaptic currents; I<sub>h</sub>, hyperpolarization-activated cation current; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogenactivated protein kinase; n.s., not significant; PC, Purkinje cell; PF, parallel fiber; PFEPCs, excitatory postsynaptic currents evoked by parallel fiber stimulation; PKC, protein kinase C.

## 1. Introduction

The serotonergic axons are widely distributed throughout the brain and modulate key aspects of physiological functions including sensory processing, cognitive control, emotion and motor activity (Meneses and Liy-Salmeron, 2012). Given the widespread innervation of the brain and the richness of signals evoked by serotonin (5-hydroxytryptamine; 5-HT), the 5-HT system has been identified as a target of many pharmacological treatments for psychiatric and neurological conditions such as depression, anxiety, schizophrenia and drug abuse (Müller and Jacobs, 2009; Olivier, 2015). Serotonergic modulation of the cerebellum has been subject of clinical interest since the discovery that long-term administration of L-5-hydroxytryptophan (5-HTP), a precursor of 5-HT, improves the dysfunctions associated with cerebellar disorders in patients with inherited or acquired ataxias (Trouillas et al, 1988 and 1995). On the other hand, there is evidence that neurodevelopmental disorders such as autism and schizophrenia are associated with a change in 5-HT receptor expression in the cerebellum (Slater et al, 1998; Eastwood et al, 2001). The cerebellum receives an intense innervation from serotonergic raphe neurons that affects all parts of the cerebellar circuitry, i.e. cerebellar cortex and nuclei (Bishop and Ho, 1985). The role of 5-HT in the cerebellum has been assumed as a regulator of sensorimotor learning. First, 5-HT release into the cerebellum is positively correlated with the level and type of motor activity (Mendlin et al, 1996). Second, chronic treatment with 5-HTP or buspirone, a 5-HT<sub>1A</sub> receptor (5-HT<sub>1A</sub>R) partial agonist, improves the motor coordination deficits in Lurcher mouse, a model of cerebellar neurodegeneration (Le Marec et al, 2001). Third, pharmacological depletion of serotonergic inputs impairs the horizontal vestibular-ocular reflex (VOR) adaptation in rabbits (Miyashita and Watanabe, 1984). However, the means by which 5-HT regulates learning processes in the cerebellum, particularly in the adult animal, remain poorly understood.

It is widely believed that synaptic plasticity in the cerebellar cortex is the physiological basis for cerebellar information learning and storage (Kandel, 2001). The best studied plastic site in the cerebellar cortex is the synapse between parallel fibers (PF) and Purkinje cells (PC), which undergoes both long-term depression (LTD) and potentiation (LTP), depending upon the combination of different afferent signals and postsynaptic activity (Ito, 2001; Hartell, 2002). Immunohistochemical studies have revealed the presence of several subtypes of 5-HT receptors at the level of Purkinje cells, which also receive significant serotonergic inputs (Geurts et al, 2002). These findings raise the possibility that the serotonergic projection to PC may modify the synaptic efficacy of excitatory inputs and thus modulate the neural circuits implicated in cerebellar dependent learning and memory. Among the 5-HT receptor subtypes potentially involved in activity-induced plasticity, the 5-HT<sub>7</sub> receptor (5-HT<sub>7</sub>R) has recently received much attention

because of its broad expression in central nervous system, specially in thalamus, hypothalamus, hippocampus, striatum and cortex (Matthys et al, 2011). Contextual learning, spatial memory and hippocampal long-term synaptic plasticity are all impaired in mice treated with selective antagonists of 5-HT7R as well as in 5-HT7R knockout (KO) mice (Roberts et al, 2004; Roberts and Hedlund, 2012). Furthermore, putative 5-HT7R-specific agonists improve cognitive functions in animal models and facilitate synaptic activity in hippocampus (Meneses et al, 2015). Although expression of 5-HT7R in Purkinje cells has been reported (Geurts et al, 2002), its role in cerebellar synaptic transmission and plasticity has not been defined. Thus, in the present study we examined the effect of a specific 5-HT7R agonist on the PF-PC synapse in cerebellar slices of adult mice. The results presented here show that activation of 5-HT7R by the selective agonist LP-211 (Hedlund et al, 2010), produces a long-lasting synaptic depression between parallel fibers and Purkinje cells. Such depression is postsynaptically expressed and requires the activation of protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) signaling pathways. Moreover, bath application of a 5-HT7R antagonist prevented LTD produced by pairing parallel fiber stimulation with Purkinje cell depolarization. On the other hand, bath application of LP-211 blocked LTP induced by 1 Hz parallel fiber stimulation. These results suggest that the activation of 5-HT7R shifts cerebellar synaptic plasticity towards depression, via PKC and MAPK pathways. Surprisingly, 5-HT7R activation was not only necessary, but also sufficient to trigger cerebellar LTD. These results might provide a basis for the development of new therapeutic agents and strategies for the treatment of learning and memory disorders involving the cerebellum.

## **2. Materials and methods**

### **2.1. Slice preparations**

Experiments were performed on parasagittal slices prepared from the cerebellar vermis of CD-1 mice of either sex, 2 - 4 months old, as previously described (Lippiello et al, 2015). Experiments were approved by the Local Ethical Committee of the University of Naples and were in agreement with the governmental guidelines. Each animal was anaesthetized with isoflurane, USP (Abbott Laboratories, Illinois, USA) and decapitated. The cerebellar vermis was removed and rapidly immersed in ice-cold extracellular saline solution containing (mM): 125 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 20 glucose and the pH was maintained at 7.4 by bubbling with 95% O<sub>2</sub> – 5% CO<sub>2</sub>. Parasagittal cerebellar slices (200 µm thickness) were obtained using a vibratome (Vibroslice 752, Campden Instruments, Loughborough, UK) and kept at room temperature (25 °C).

## 2.2. Electrophysiology

Individual slices were transferred to a recording chamber and continuously perfused with the extracellular solution at a rate of 1.5 ml/min using a peristaltic pump. The PC soma was visually identified using a 40x water-immersion objective of an upright microscope (BX50WI, Olympus, Japan). Pipettes of borosilicate glass, with a tip diameter of 2–3  $\mu\text{m}$  and a resistance between 2 and 3  $\text{M}\Omega$ , were used for patch-clamp recording. The electrodes were filled with an internal solution containing (mM): 130 potassium gluconate, 8 KCl, 2  $\text{MgCl}_2$ , 10 Hepes, 4  $\text{Na}_2\text{ATP}$ , 0.4  $\text{Na}_3\text{GTP}$ , 10 EGTA; the pH was adjusted to 7.3 with 1M KOH (Lippiello et al., 2015). For the climbing fiber stimulation experiments, the internal solution contained  $\text{Cs}^+$  to block voltage-gated  $\text{K}^+$  channels and consisted of: 134 CsCl, 4  $\text{MgCl}_2$ , 10 Hepes, 4  $\text{Na}_2\text{ATP}$ , 0.4  $\text{Na}_3\text{GTP}$ , 10 EGTA; the pH was adjusted to 7.3 with 1M CsOH. Indeed, climbing fiber-evoked EPSCs are generally larger (greater than 2 nA) than parallel fiber evoked EPSCs and therefore capable of activating voltage-dependent conductances in Purkinje cells. For the LTD experiments, the internal solution consisted of (mM): 130 potassium gluconate, 2 NaCl, 4  $\text{MgCl}_2$ , 4  $\text{Na}_2\text{ATP}$ , 0.4 NaGTP, 20 HEPES, 0.25 EGTA. All recordings were performed in whole-cell configuration, using an EPC-8 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Voltage-clamp recordings were accepted only if the series resistance was less than 10  $\text{M}\Omega$ . The holding potential was set at  $-60$  mV. Data were filtered at 3 kHz, digitized at 10 kHz and stored using the Pulse software (HEKA Elektronik). A soda lime glass pipette (tip diameter 10–15  $\mu\text{m}$ , filled with extracellular saline solution) was placed in the molecular layer or granular layer to stimulate parallel fibers or climbing fibers, respectively. The fibers were stimulated with pairs of stimuli separated by 100 ms, delivered every 20 seconds by an isolated stimulator (A/M Systems, Carlsborg, WA, USA). Drugs were applied by switching the perfusion from control saline to drug-containing saline without altering the perfusion rate. The time required for the drug solution to reach the recording chamber was about 90 s, which was indicated as time 0. Gabazine (20  $\mu\text{M}$ ) was added in the bath throughout the recording to block GABAA receptors. LP-211 (N-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide) was synthesized at the Department of Pharmacy, University of Bari "A. Moro", Italy, as previously described (Leopoldo et al, 2008). SB-269970 and WAY 100635 maleate were purchased from Tocris Bioscience (Bristol, UK). Gabazine, 5-HT and kynurenic acid were purchased from Abcam (Cambridge, UK). Ketanserin and GF109203X were from Sigma-Aldrich (Milan, Italy); U0126 was from Cell Signaling (Danvers, MA, USA). GF109203X and U0126 were dialyzed intracellularly via the patch pipette for at least 30 min before LP-211 application.

### **2.3. Immunohistochemistry**

Adult mice (2 - 4 months old) were anesthetised (as above) and perfused transcardially with 20 mL PBS followed by 30-40 mL freshly prepared fixative (4% paraformaldehyde dissolved in PBS, pH 7.4). The cerebellum was dissected and post-fixed for 24 h at 4°C and then cryoprotected overnight in PBS containing 30% sucrose. Tissues were embedded in O. C. T compound (Bio-Optica, Milan, Italy), cut on a cryostat and processed. Sagittal cerebellar slices (50 µm thickness) were permeabilized for 20 min at RT in PBS containing 0.1% Triton-X-100 and 10% normal goat serum (NGS, G90-23, Sigma Aldrich, Milan, Italy) and then blocked with PBS, 10% NGS and 0.1% bovine serum albumine (BSA) for 1 hr at RT. The slides were incubated in PBS and 0.1% BSA at 4°C for 48 h with the primary antibodies (anti-rabbit 5-HT7R receptor, 1:50, Imgenex, Milan Italy; anti-mouse calbindin, 1:1500, Sigma Aldrich). After rinsing with PBS, the fluorescent-labeled secondary antibodies (Alexa 488 anti-rabbit, 1:200, Invitrogen, Life Technologies, Milan, Italy; Alexa 594 anti-mouse, 1:200, Invitrogen, Life Technologies, Milan, Italy) were added for 2 h. Control slides were incubated in the same solutions without primary antibodies and subsequently processed as above. Images were acquired with a Leica microscope (Leica DM6000B) equipped with a 20x objective. For quantification of double-immunoreactive cells, images were acquired with high-resolution camera using the software Leica Application Suite, and were analyzed by the image-processing software Image J. The images were pre-processed to optimize illumination and contrast. The number of calbindin- and 5-HT7R-immunopositive cells was manually estimated using the "Multi-point selections" function of the software.

### **2.4. Statistics**

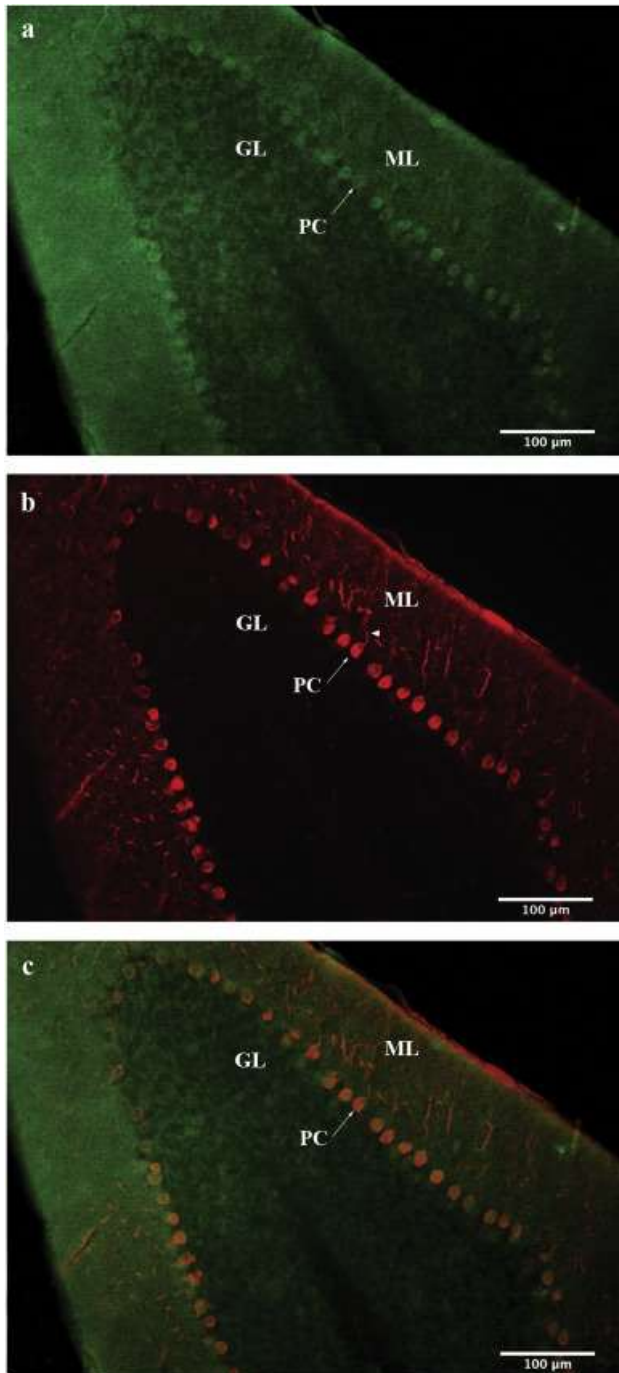
Data analysis was performed with Excel (Microsoft) and GraphPad Prism 5 (San Diego, CA, USA). For each cell, the EPSC amplitude during and after drug applications was normalized to the mean EPSC amplitude during 10-min baseline recording. Drug effects were expressed as % change (mean  $\pm$  SEM) from a pre-drug control baseline. Paired or unpaired two-tailed Student's t-test evaluated the statistical significance of various drug effects (% change from control baseline).

## **3. Results**

### **3.1. Localization of 5-HT7R in the mouse cerebellar cortex**

To characterize the distribution of 5-HT7R in the cerebellar cortex, we performed immunostaining of cerebellar sagittal slices using a specific antibody against the 5-HT7R. To detect PCs, we used

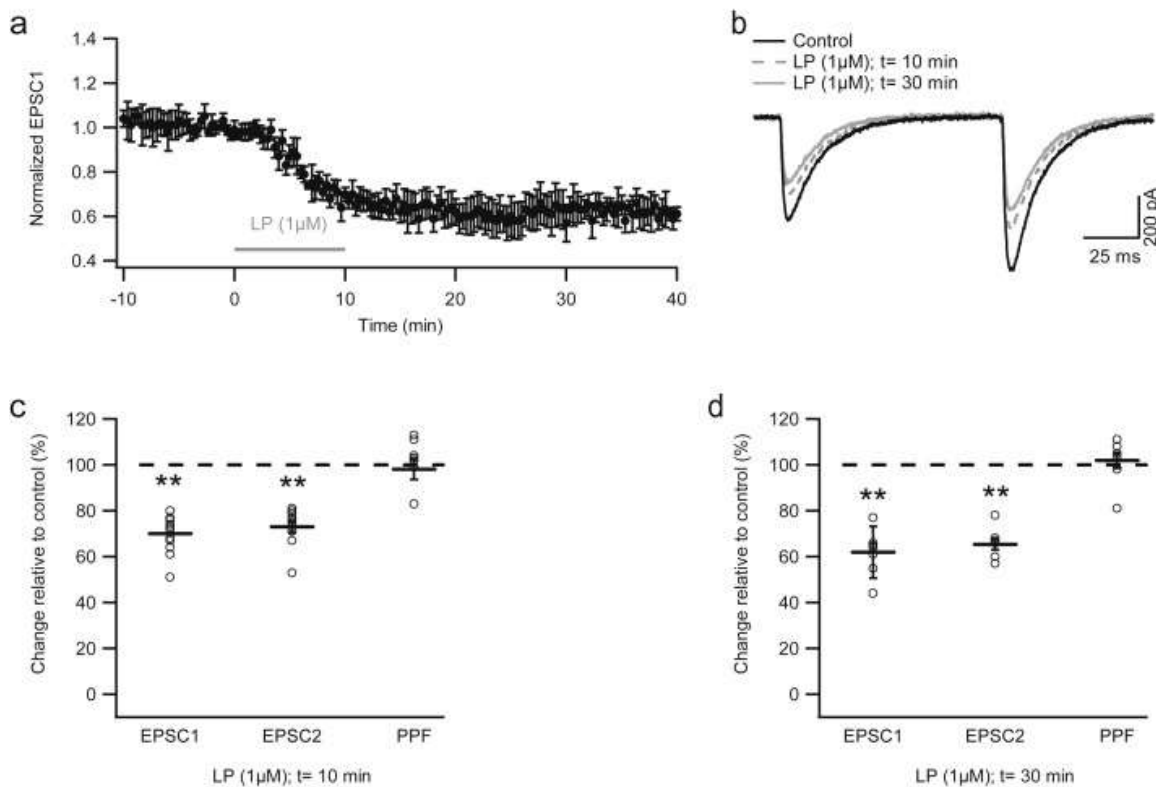
the  $\text{Ca}^{2+}$ -binding protein calbindin, which is highly expressed in PCs. As shown in Figure 1, 5-HT7R was strongly expressed in the soma and dendrites of Purkinje cells; the quantification revealed that  $85 \pm 1.9\%$  of calbindin-positive cells colocalized with 5-HT7R. A diffuse immunoreactivity for the 5-HT7R was also observed in the molecular layer and to a lesser extent in the granular cell layer.



**Figure 1.** Expression of 5-HT7R in the mouse cerebellar cortex. (a) Immunofluorescent detection showing expression of 5-HT7R in Purkinje cell (PC; arrow); granular layer (GL) and molecular layer (ML). (b) Identification of PCs using anti-calbindin antibody. Purkinje cell soma (arrow) and dendrites (arrowheads) are indicated. (c) 5-HT7R and calbindin co-localization. Scale bar = 100 μm.

### 3.2. Activation of 5-HT7R causes long-lasting depression of the EPSC evoked by PF stimulation

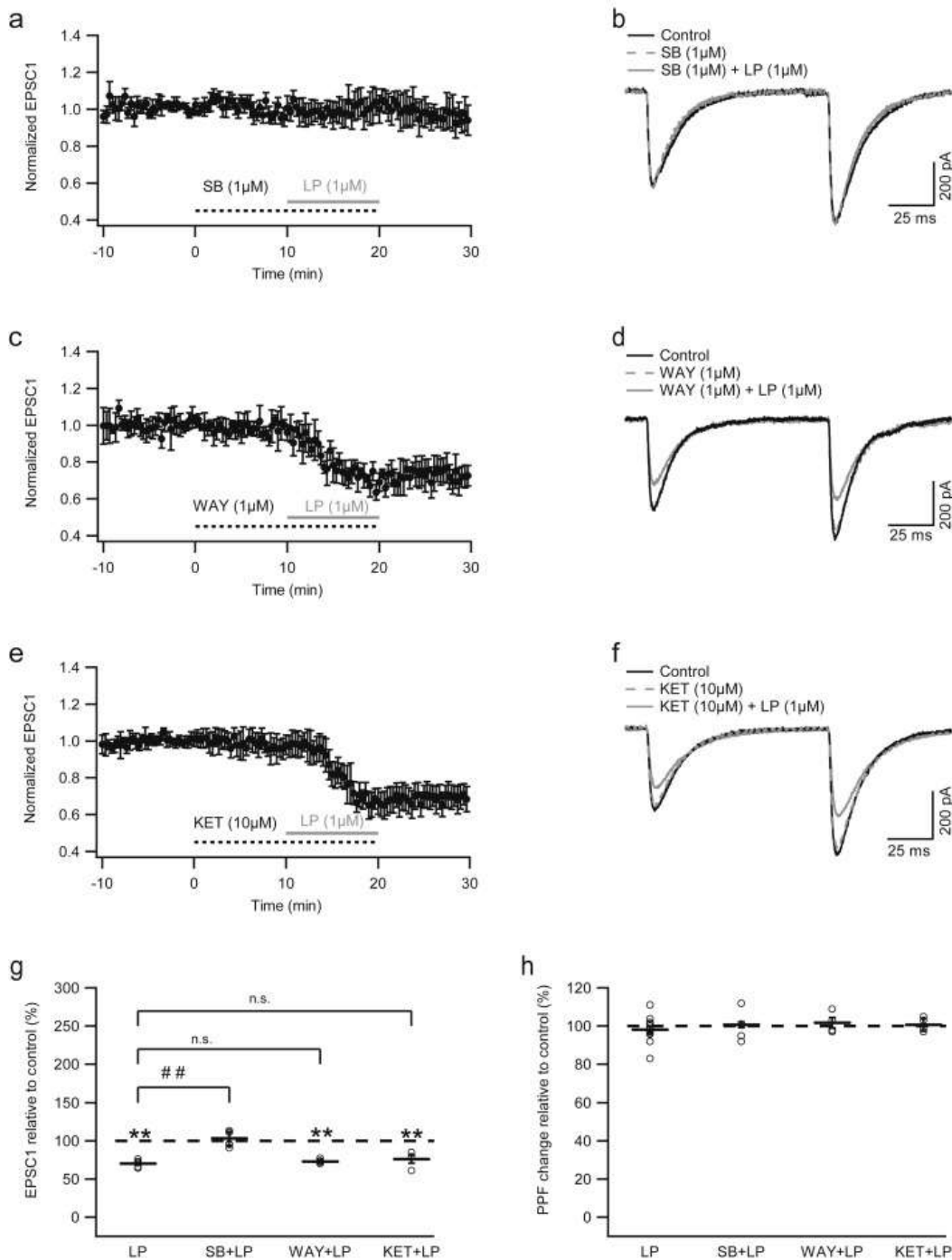
To determine whether the activation of 5-HT7R plays a role in modulation of the PF-PC synapse, we examined the effect of the selective 5-HT7R agonist LP-211, on the excitatory postsynaptic currents (EPSCs) evoked by parallel fiber stimulation. As shown in Figure 2, LP-211 (1  $\mu$ M) decreased the EPSC1 amplitude by  $29.9\% \pm 2.2$  after 10 min application ( $n = 12$ ;  $p < 0.01$  vs control) and the depression persisted after washout of the drug, throughout the duration of the experiment. Thirty minutes after washout of LP-211, EPSC1 was still depressed by  $39.2\% \pm 11.2$  ( $p < 0.01$  vs control). In order to determine whether LP-211 acts on presynaptic or postsynaptic components of PF-PC synapses, we analyzed the paired-pulse ratio before and after perfusion with LP-211. Figure 2c and Figure 2d show that the PPF ratio remained unchanged after perfusion with LP-211 (n.s. vs control) suggesting that LP-211-induced decrease in EPSC amplitude involves modifications of postsynaptic components of the PF-PC synapse.



**Figure 2.** LP-211 induces long-term depression of PF-PC synapse through activation of 5-HT7R. (a) Time course of average PF-EPSC1 amplitude ( $\pm$  SEM;  $n = 12$ ), normalized by baseline values. LP-211 (LP, 1  $\mu$ M) was bath applied during the time indicated by the bar. (b) Representative PF-EPSC traces evoked by paired pulse stimulation before (control) and after (10 and 30 min) LP application. (c-d) Scatter plots showing the effect of LP on EPSC1, EPSC2 and paired pulse facilitation (PPF) at 10 min or 30 min after LP application relative to the control (before LP application). Horizontal lines represent the mean percentage of change ( $\pm$  SEM,  $n = 12$ ) after LP application relative to the control (before LP application). Data points are presented as dots. Asterisks indicate statistical difference from the control (\*\* $p < 0.01$ , Student's t-test).



To further confirm the involvement of 5-HT<sub>7</sub>R in the modulation of PF-PC synaptic response by LP-211, we examined the effect of the selective 5-HT<sub>7</sub>R antagonist SB-269970 (1  $\mu$ M) added 10 min before and during LP-211 (1  $\mu$ M) application. SB-269970 completely suppressed the LP-211-induced depression of EPSC to values similar to control ( $n = 5$ ,  $p = \text{n.s.}$  vs control;  $p < 0.01$  vs LP-211; Figures 3a, b, g and h), showing that 5-HT<sub>7</sub>R is responsible of the effect of LP-211 on PF-PC synapse.

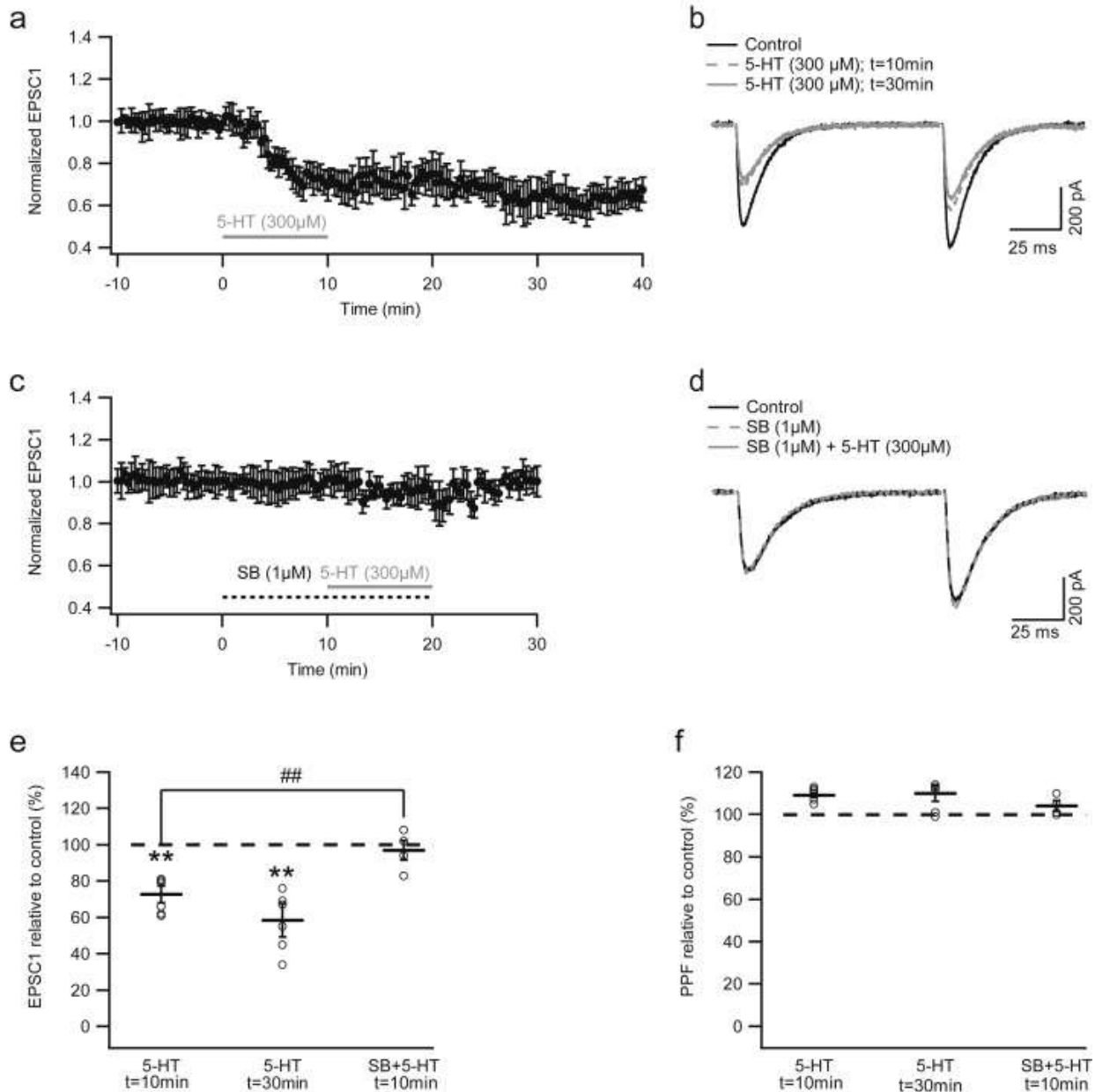


**Figure 3.** 5-HT<sub>7</sub>R selective antagonist abolishes the effect of LP-211 at the PF-PC synapse. (a) Effects of LP-211 (LP, 1  $\mu$ M) on normalized PF-EPSC1 amplitude (mean  $\pm$  SEM,  $n = 5$ ) in the presence of the selective 5-HT<sub>7</sub>R antagonist SB-269970 (SB, 1  $\mu$ M). (b) Sample PF-EPSCs taken in control condition and in the presence of SB and LP (c) Effects of LP (1  $\mu$ M) on normalized PF-EPSC1 (mean  $\pm$  SEM,  $n = 4$ ) in the presence of the 5-HT<sub>1A</sub>R antagonist WAY 100635

(WAY, 1  $\mu$ M). (d) Superimposed PF-EPSCs recorded in control condition and in the presence of LP and WAY. (e) Effects of LP (1  $\mu$ M) on normalized PF-EPSC1 (mean  $\pm$  SEM,  $n = 4$ ) in the presence of the 5-HT<sub>2R</sub> antagonist ketanserin (KET, 10  $\mu$ M). (f) Superimposed PF-EPSCs in control condition and in the presence of LP and KET. (g-h) Scatter plots of the mean (horizontal lines;  $\pm$  SEM) percentage of change of PF-EPSC1 amplitude and PPF ratio relative to the control measured at 10 min after application of LP alone or in combination with 5-HT<sub>7R</sub>, 5-HT<sub>1AR</sub> or 5-HT<sub>2R</sub> antagonist (\*\*  $p < 0.001$  vs control). The ## symbol indicates  $p < 0.01$  vs LP (Student's t-test).

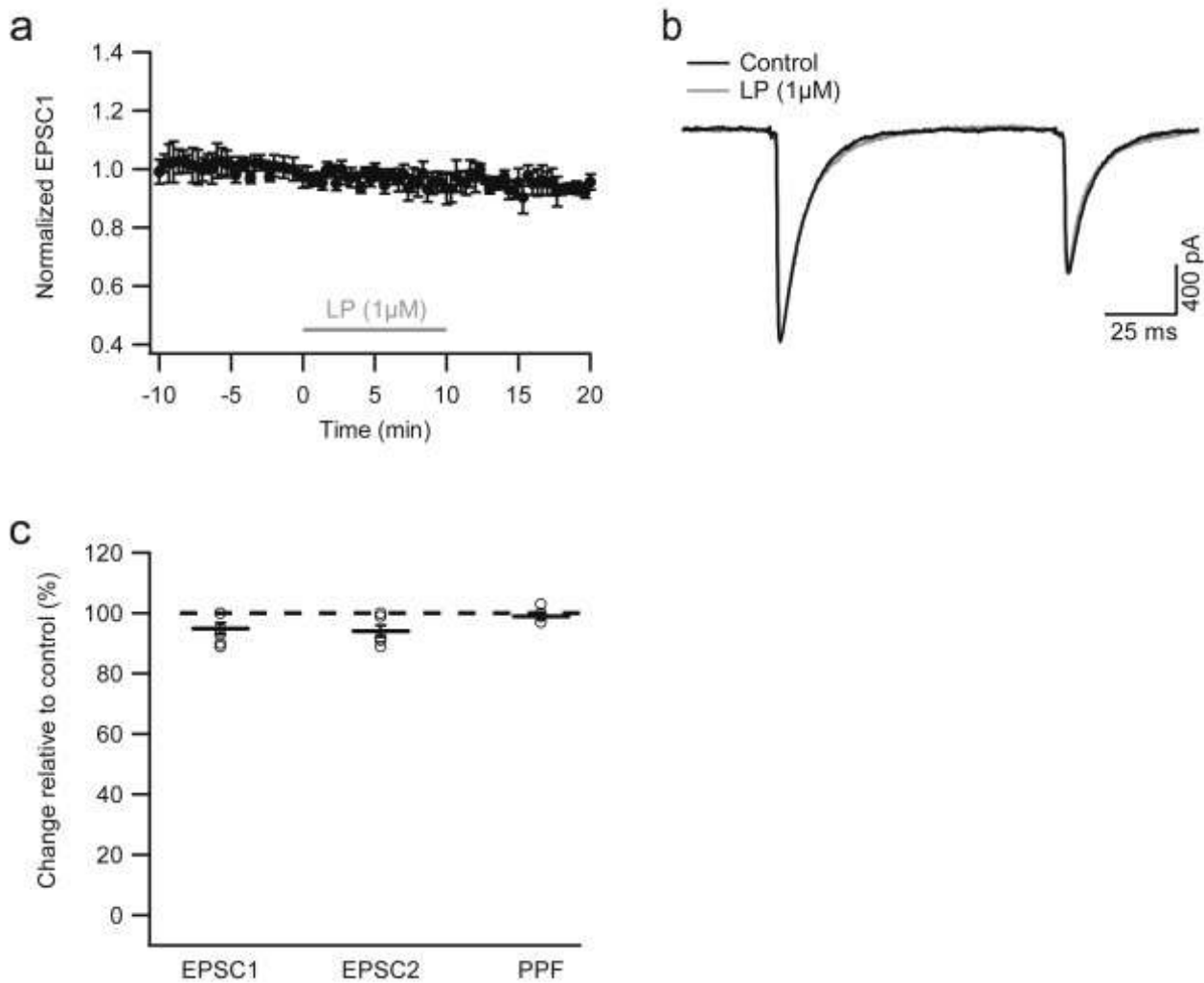
Since both 5-HT<sub>1A</sub> and 5-HT<sub>2R</sub>s are expressed in mouse cerebellum, predominantly in PC and molecular layers (Geurts et al, 2002; Li et al, 2004), we performed experiments in which LP-211 was applied in the presence of either a 5-HT<sub>1A</sub> (WAY 100635) or a 5-HT<sub>2A-C</sub> receptor (ketanserin) antagonist to further establish the pharmacological specificity of LP-211 effect. Both WAY 100635 (1  $\mu$ M;  $n = 4$ ; Figures 3c, d, g and h) and ketanserin (10  $\mu$ M;  $n = 4$ ; Figures 3e, f, g and h) did not affect the decrease in PF-EPSC observed in the presence of LP-211 ( $p < 0.01$  vs control;  $p = \text{n.s.}$  vs LP-211) showing that 5-HT<sub>1A</sub> and 5-HT<sub>2</sub> receptors do not participate in the LP-211-induced depression of PF-EPSCs.

To determine whether 5-HT<sub>7R</sub>s contribute to the synaptic response to 5-HT, the physiological neurotransmitter released by serotonergic fibers, we examined the modulatory effect of 5-HT on the PF-PC synapse in the presence of the 5-HT<sub>7R</sub> antagonist SB-269970. We found that 5-HT (300  $\mu$ M) produced a significant depression of the PF-EPSC that did not recover following 5-HT washout ( $-27\% \pm 4.7\%$  at  $t = 10$  min and  $-41.5\% \pm 9.2$  at 30 min,  $n = 6$ ;  $p < 0.01$  vs control; Suppl. Fig. S1) and did not alter the PPF ratio ( $p = \text{n.s.}$  vs control). The 5-HT effect was significantly counteracted by application of the 5-HT<sub>7R</sub> antagonist SB-269970 (1  $\mu$ M;  $n = 4$ ,  $p = \text{n.s.}$  vs control;  $p < 0.01$  vs 5-HT), indicating that the 5-HT-induced depression of PF-PC synapses was mostly mediated by 5-HT<sub>7R</sub>s.



**Figure 4.** 5-HT induces depression of PFePC synapse through the activation of 5-HT7Rs. (a) Time course of normalized PF-EPSC1 amplitude (mean ± SEM; n = 6) in the presence of 5-HT (300 μM). (b) Superimposed traces of EPSCs recorded from the PC before (control) and after (10 min or 30 min) 5-HT application. (c) Effects of 5-HT (300 μM) on normalized PFEPSC1 amplitude (mean ± SEM, n = 4) in the presence of the selective 5-HT7R antagonist SB-269970 (SB, 1 μM). (d) Sample PF-EPSCs recorded in control condition and in the presence of SB and 5-HT. (e) Scatter plots of the mean (horizontal lines; ± SEM) percentage of change of PF-EPSC1 amplitude relative to the control measured after application of 5-HT alone (at 10 min or 30 min) or in combination with 5-HT7R antagonist (at 10 min; \*\*p < 0.01 vs control). The ## symbol indicates p < 0.01 vs 5-HT (Student's t-test). (f) Scatter plots of the mean (horizontal lines; ± SEM) percentage of change of PPF ratio relative to the control measured after application of 5-HT alone (at 10 min or 30 min) or in combination with 5-HT7R antagonist (at 10 min).

We then asked whether the modulation of PF-EPSCs by LP-211 was specific to parallel fiber, or whether climbing fiber-Purkinje cell synapses were also affected. We found that LP-211 did not alter the amplitude or paired-pulse ratio of CF-EPSCs (n = 6, p = n.s. vs control; Figure 5).

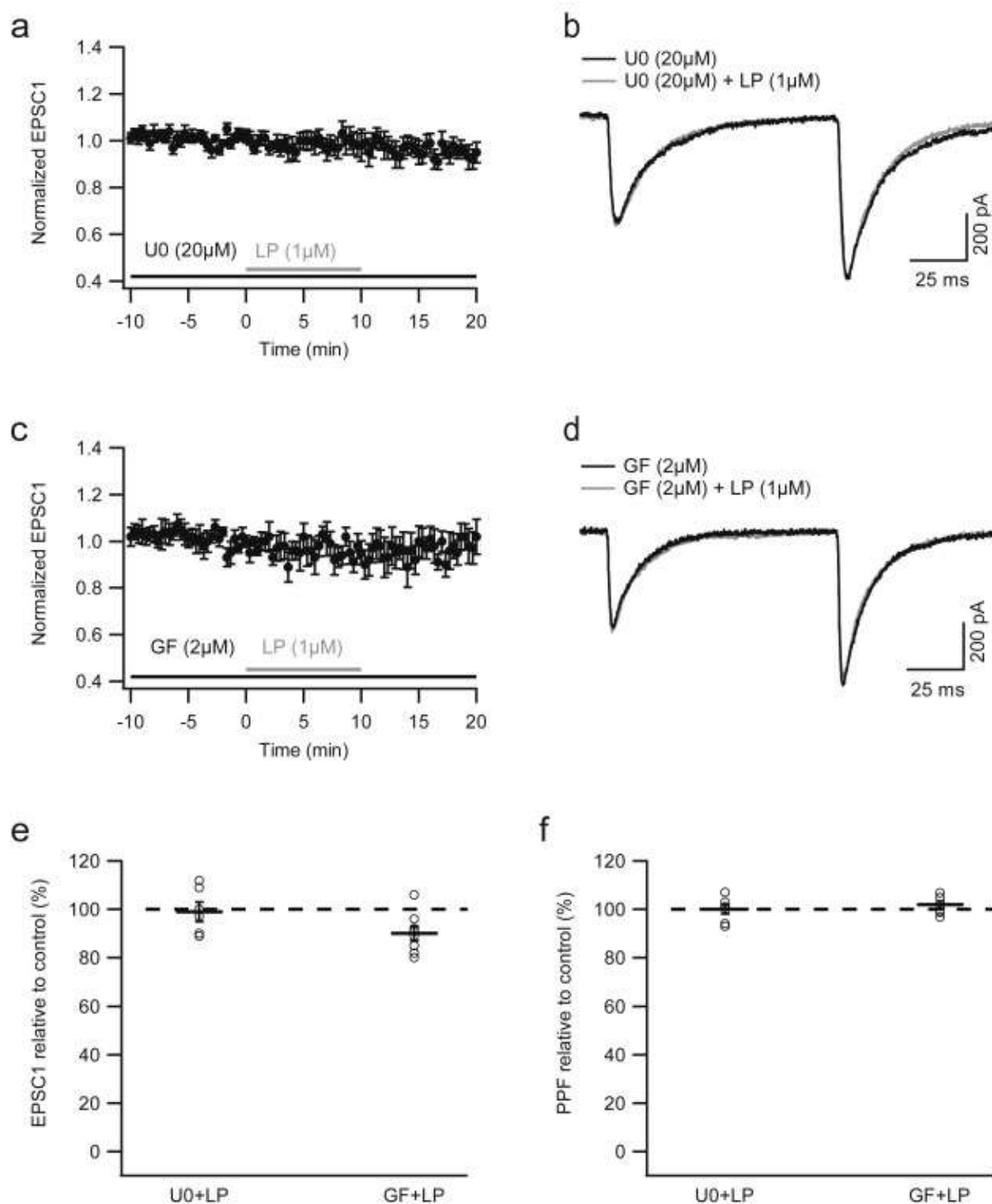


**Fig. 5.** LP-211 does not affect the climbing fiber to Purkinje cell synapse. (a) Time course of normalized CF-EPSC1 (mean  $\pm$  SEM;  $n = 6$ ) in PCs in response to pairs of stimuli. LP-211 (LP, 1  $\mu$ M) was bath applied during the time indicated by the bar. (b) Sample CF-EPSC traces recorded before (control) and during LP application. (c) Scatter plot showing the effect of LP on CF EPSC1, EPSC2 and paired pulse facilitation (PPF). Horizontal lines represent the mean percentage of change ( $\pm$ SEM,  $n = 6$ ) at 10 min after LP application relative to the control (before LP application).

To ensure that the different effect of LP on CF-PC and PF-PC synapses was not due to the different internal solutions, we performed a series of PF-EPSCs recordings where the Purkinje cells were patched with a CsCl based internal solution. Under this condition, LP-211 (1  $\mu$ M) still decreased the PF-EPSC1 amplitude by  $30.5\% \pm 2.9$  after 10 min application ( $n = 8$ ;  $p < 0.01$  vs. control) and the depression persisted after washout of the drug, throughout the duration of the experiment (data not shown).

### **3.3. The LP-211-induced depression of PF–PC synaptic transmission is mediated by the PKC-MAPK dependent pathway**

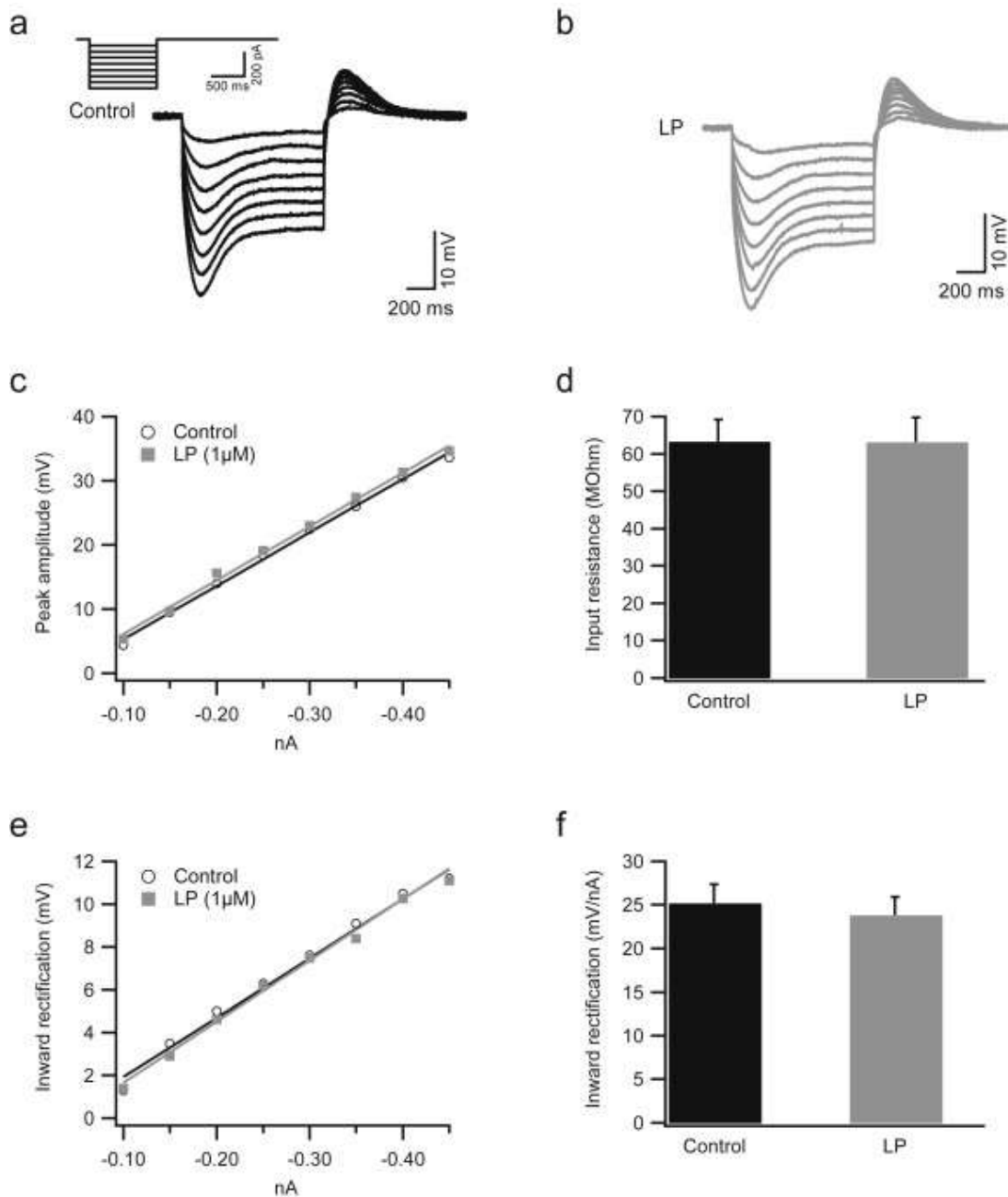
5-HT<sub>7</sub>R<sub>s</sub> are G<sub>s</sub> protein-coupled receptors that stimulate adenylate cyclase resulting in an increased cAMP production and activation of protein kinase A (Guseva et al., 2014). cAMP causes in turn activation of MAPK such as the extracellular-regulated kinase (ERK1/2; Lin et al., 2003). In addition, there is evidence that 5-HT<sub>7</sub>R can also activate PKC indicating that the same receptor may contribute to different signaling pathways besides the canonical PKA downstream pathway (Lieb et al., 2005). Interestingly, both MAPKs and PKC have been shown to play critical roles in synaptic plasticity, including LTD in cultured Purkinje cells and in cerebellar slice preparations (Kawasaki et al., 1999, Endo and Launey, 2003 and Ito-Ishida et al., 2006). According to recent studies, MAPK activation requires PKC and triggers a positive feedback responsible for the sustained activation of PKC, which is required for the maintenance of LTD (Tanaka and Augustine, 2008). We therefore examined the possible involvement of MAPKs in the LP-211-mediated depression of PF–PC synapse by infusing a MAP kinase inhibitor U0126 (20  $\mu$ M) into Purkinje cells via patch pipette. In the presence of U0126, LP-211 failed to depress the PF–PC synapses suggesting that MAPK is enrolled by 5-HT<sub>7</sub>R ( $n = 9$ ,  $p = \text{n.s.}$  vs. control; Fig. 6a, b and e). We next examined whether PF-EPSC depression produced by LP-211 could be also blocked by the PKC inhibitor GF 109203X (2  $\mu$ M), added in the patch pipette. GF treatment prevented the inhibitory action of LP-211 on the PF-EPSCs ( $n = 8$ ,  $p = \text{n.s.}$  vs. control; Fig. 6c–e). The introduction of GF109203X or U0126 cells into Purkinje cells did not alter the PPF ratio, thus confirming the postsynaptic site of action of these inhibitors (Fig. 6f;  $p = \text{n.s.}$  relative to control). Altogether, these results demonstrate the critical role of both MAPK and PKC as downstream effectors of 5-HT<sub>7</sub>R<sub>s</sub> in the depression of excitatory transmission between PF and PC.



**Fig. 6.** Effect of protein kinase C and MAPK inhibitors on the 5-HT<sub>7</sub>R-induced depression of PF-EPSCs. (a) Time course of normalized PF-EPSCs (mean  $\pm$  SEM;  $n = 9$ ) before, during and after a 10-min application of LP-211 (LP, 1  $\mu$ M), in the presence of the MAP kinase inhibitor U0126 (U0, 20  $\mu$ M) in the patch-pipette. (b) Superimposed PF-EPSCs recorded in control condition and during application of LP plus U0. (c) Time course of normalized PF-EPSCs (mean  $\pm$  SEM;  $n = 8$ ) recorded during application of LP-211 in the presence of the PKC inhibitor GF 109203X (GF, 2  $\mu$ M) in the patch-pipette. (d) Sample PF-EPSCs taken from a PC loaded with GF 109203X, before and during treatment with LP. (e-f) Scatter plots of PF-EPSC1 and PPF ratio during application of LP either with U0126 ( $n = 9$ ) or GF 109203X ( $n = 8$ ) in the patch-pipette. Horizontal lines represent the mean percentage of change ( $\pm$ SEM) relative to control.

### **3.4. 5-HT7R activation does not affect the hyperpolarization-activated cation current**

Previous electrophysiological studies have shown that stimulation of 5-HT7R elicits a slow membrane depolarization in neurons of brain regions where these receptors are highly expressed i.e. thalamus, hippocampus, striatum and anterior cingulate cortex (Chapin and Andrade, 2001, Bonsi et al., 2007 and Santello and Nevian, 2015). Such a depolarizing response has been attributed to the ability of 5-HT7R to activate a hyperpolarization-activated cation current (I<sub>h</sub>) via a cAMP-dependent mechanism. The increase of I<sub>h</sub> has been suggested to have a “paradoxical” inhibitory effect on the neuronal excitability and synaptic responsiveness (George et al., 2009). To examine whether 5-HT7R influences I<sub>h</sub> in Purkinje cells, we carried out whole-cell current clamp recordings of membrane properties in the presence of kynurenic acid to block glutamatergic transmission. In response to hyperpolarizing step currents injected in the Purkinje cell soma, the membrane voltage showed a depolarizing sag that is characteristic of I<sub>h</sub> activation. The sag was eliminated by the addition of a selective I<sub>h</sub> blocker ZD7288 (20  $\mu$ M) to the bath solution (data not shown). As shown in Fig. 7, bath application of LP-211 had no detectable effect on I<sub>h</sub> properties and its voltage dependence. This result excludes any contribution of I<sub>h</sub> in the inhibitory effect of LP-211 on the PF–PC synapse.

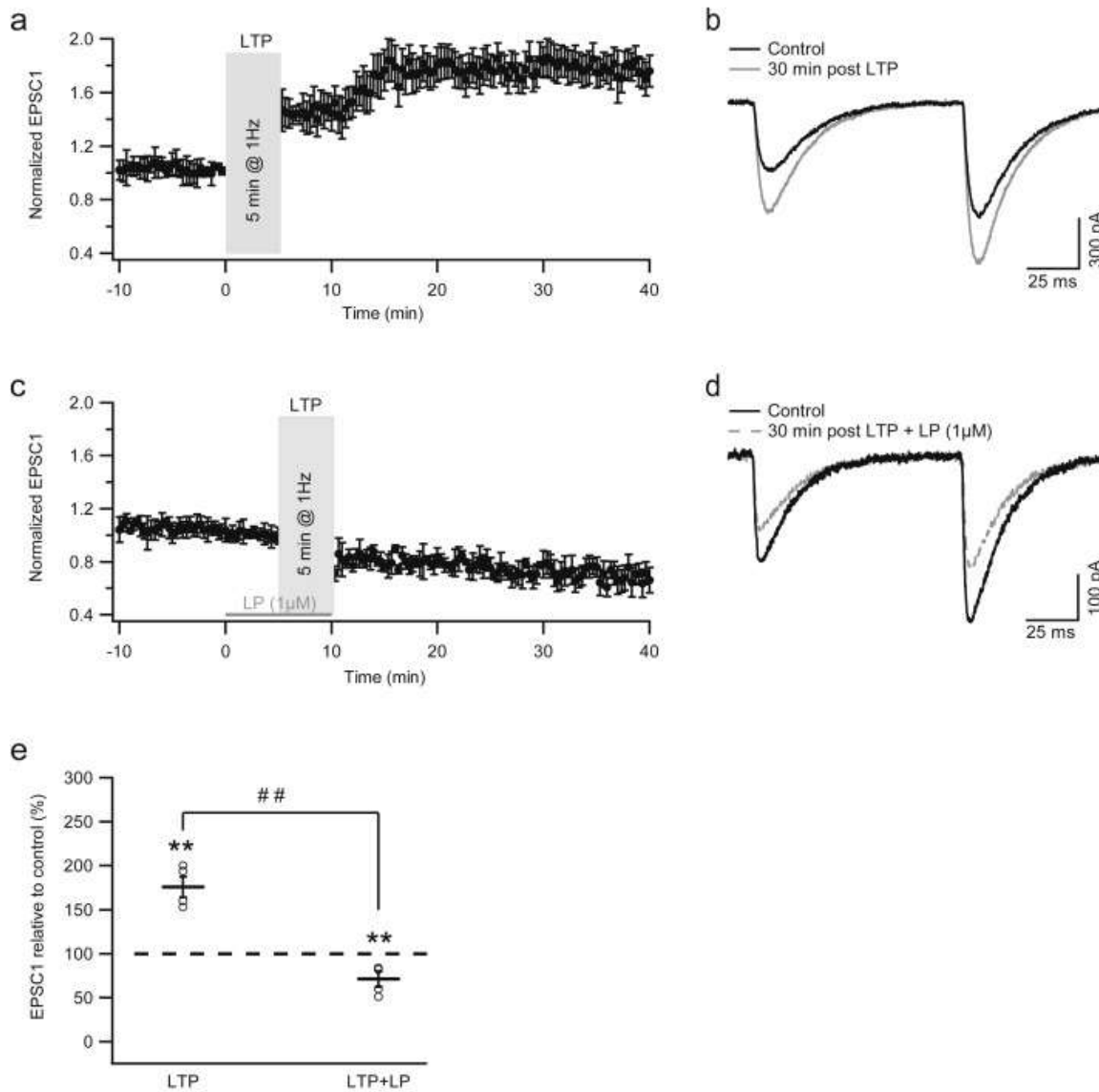


**Fig. 7.** 5-HT<sub>7</sub>R does not modulate the hyperpolarization-activated current ( $I_h$ ) in Purkinje cells. Response of PCs to hyperpolarizing current steps (ranging from  $-100$  to  $-450$  pA, in  $50$  pA steps) under control condition (a) and in the presence of LP-211 (LP,  $1$   $\mu$ M) (b). (c) Example of plots of inward rectification versus injected hyperpolarizing currents. The peak amplitude corresponded to the maximal negative deflections from the baseline. (d) Mean ( $\pm$ SEM) input resistance values in control condition and after  $10$  min of LP treatment ( $n = 7$ ;  $p = \text{n.s.}$ ). Input resistance was determined by the slope of a best-fit line of the voltage–current plot. (e) Example of plots of inward rectification versus injected currents. The inward rectification was measured as the difference between the maximum negative voltage deflection and the stable voltage level reached during the hyperpolarizing. (f) Mean ( $\pm$ SEM) inward rectification in control condition and after  $10$  min of LP treatment ( $n = 7$ ;  $p = \text{n.s.}$ ).



### 3.5. 5-HT7R modulates synaptic plasticity at parallel fiber–Purkinje cell synapses

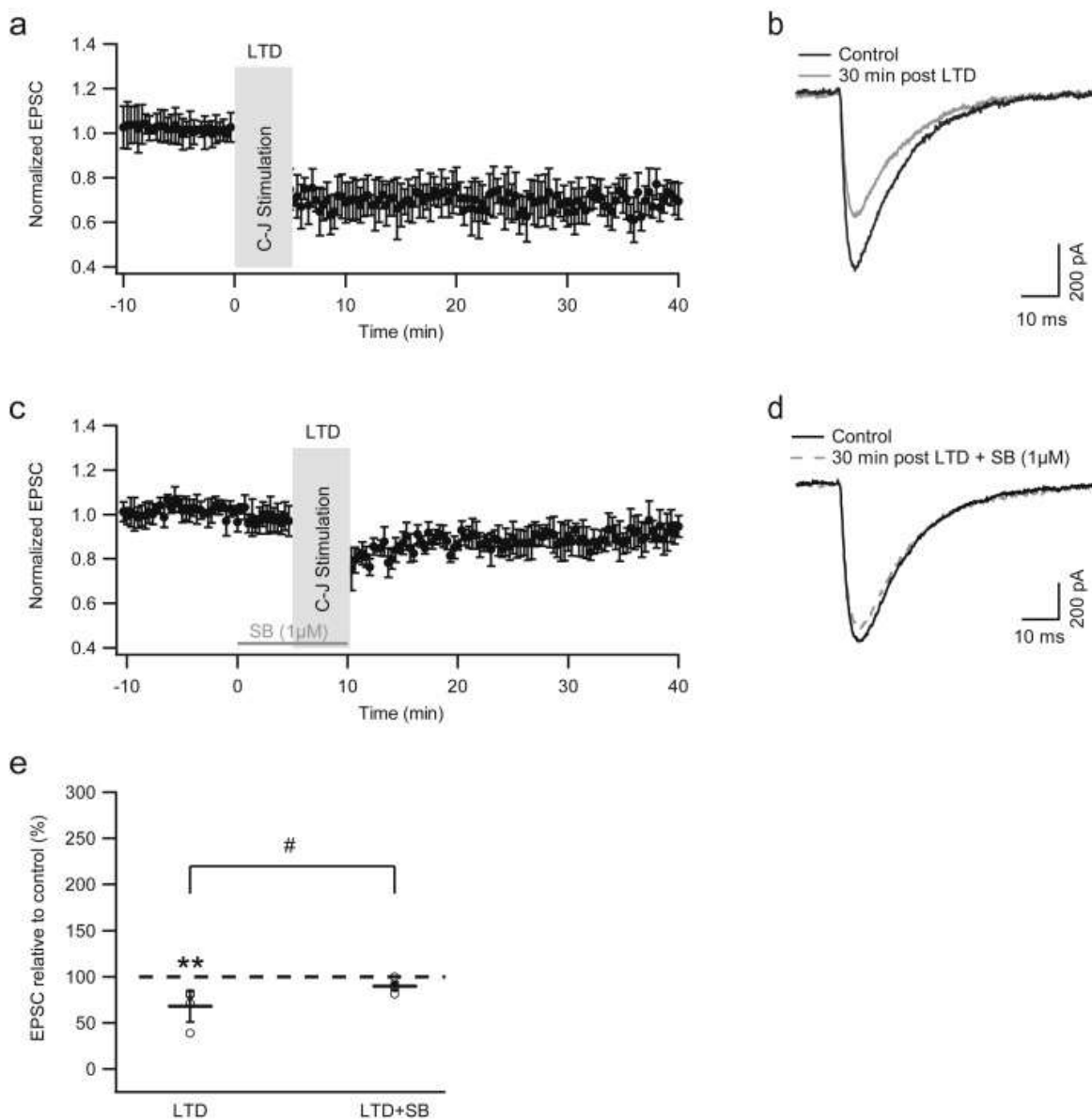
5-HT7R is considered to play an important role in the maintenance and regulation of balance between potentiation and depression of hippocampal networks (Kobe et al., 2012 and Clark and Normann, 2008). In particular, it has been shown that the stimulation of 5-HT7R results in a marked enhancement of spontaneous neuronal activity leading to saturation of LTP and thus preventing further potentiation. On the other hand, 5-HT7R activation reverses the mGluR-LTD in CA3-CA1 synapse in mouse hippocampal slices by preventing the mGluR-induced endocytosis of AMPA receptors (Costa et al., 2012). Having shown that 5-HT7R activation results in long-lasting depression of PF–PC synapses, we next assessed whether such depression affects the expression and/or maintenance of postsynaptic forms of PF–PC synaptic plasticity, i.e. LTP and LTD, evoked by specific electrical stimulation protocols (Lev-Ram et al., 2002, Hirono et al., 2001 and Kakegawa et al., 2011). PF-LTP was induced by stimulating the PF input at 1 Hz for 5 min. Under control conditions, this protocol resulted in a significant increase of PF-EPSC1 amplitude ( $+76\% \pm 11\%$ ;  $n = 4$ ;  $t = 30$  min;  $p < 0.01$  vs control) without any change in PPF (Fig. 8a, b and e). Application of LP-211 ( $1 \mu\text{M}$ ) prevented the induction of LTP causing a reduction of PF-EPSC1 of  $31\%$  ( $\pm 9\%$ ;  $n = 4$ ;  $t = 30$  min;  $p < 0.01$  vs. LTP alone; Fig. 8c–e).



**Fig. 8.** 5-HT7R activation prevents LTP induction at PF-PC synapses (a) Time course of normalized PF-EPSC1 amplitude (mean  $\pm$  SEM;  $n = 4$ ) before and after LTP induced by tetanic stimulation of PF (1 Hz for 5 min). (b) Representative PF-EPSCs taken before (control) and after (30 min) 1 Hz stimulation of PF. (c) Time course of normalized PF-EPSC1 (mean  $\pm$  SEM;  $n = 4$ ) before and after tetanic stimulation of PF (5 min at 1 Hz) in the presence of LP-211 (LP, 1  $\mu$ M). (d) Sample PF-EPSCs recorded before (control) and after (30 min) 1 Hz stimulation of PF plus LP. (e) Scatter plot showing the average (horizontal lines;  $\pm$  SEM) values of PF-EPSC1 30 min after the LTP stimulation protocol in control condition (LTP;  $n = 4$ ) and in the presence of LP (LTP + LP;  $n = 4$ ), expressed as percentage relative to control (\*\* $p < 0.01$  vs. control). The ## symbol indicates  $p < 0.01$  vs. LTP (Student's t-test).

In the next set of experiments, LTD at PF-PC synapses was induced by application of a conjunctive (CJ) stimulation protocol consisting of PC depolarization (200 msec,  $-60$  to  $+20$  mV) paired with PF stimulation (300 times at 1 Hz). Under control conditions, PF-EPSC amplitudes were reduced by 30% ( $\pm 13\%$ ;  $n = 4$ ;  $t = 30$  min;  $p < 0.01$  vs. control; Fig. 9a, b and e). In the presence of the 5-HT7R antagonist SB-269970, the LTD-induction protocol reduced the mean EPSC amplitude by

only 10% ( $\pm 3.1\%$ ;  $n = 5$ ,  $t = 30$  min;  $p < 0.05$  vs. LTD alone (Fig. 9c–e) demonstrating that the inactivation 5-HT<sub>7</sub>R interferes with the expression of LTD at PF–PC synapses. Altogether, these findings indicate that modulation via 5-HT<sub>7</sub>R regulates the ability of PF–PC to undergo synaptic plasticity.



**Fig. 9.** 5-HT<sub>7</sub>R activation is required for the expression of LTD of PF–PC induced by pairing PF stimulation with PC depolarization. (a) Time course of normalized PF-EPSC (mean  $\pm$  SEM;  $n = 4$ ) before and after LTD induction. LTD was induced by application of a conjunctive (CJ) stimulation protocol consisting of PC depolarization (200 msec,  $-60$  to  $+20$  mV) paired with PF stimulation (300 times at 1 Hz). (b) Representative PF-EPSC recordings taken before (control) and after (30 min) conjunctive (CJ) stimulation protocol. (c) Time course of normalized PF-EPSC (mean  $\pm$  SEM;  $n = 5$ ) recorded before and following application of a conjunctive (CJ) stimulation protocol in the presence of the 5-HT<sub>7</sub>R antagonist SB-269970 (SB, 1  $\mu$ M). (d) Representative recordings before (control) and after (30 min) conjunctive (CJ) stimulation protocol plus SB. (e) Scatter plot showing the average (horizontal lines;  $\pm$  SEM) values of PF-EPSC 30 min after LTD stimulation protocol in control condition ( $n = 4$ ) and in the presence of SB ( $n = 5$ ), expressed as percentage relative to control (\*\* $p < 0.01$  vs. control). The # symbol indicates  $p < 0.05$  vs. LTD (Student's  $t$ -test).

## 4. Discussion

Although previous studies have shown that 5-HT induces depression of excitatory transmission (Lee et al., 1985 and Hicks et al., 1989), it was still unclear what type of 5-HT receptor was responsible for this effect, likely due to the lack of specific agonist and antagonists targeting the numerous 5-HT<sub>R</sub> subtypes. By using a specific 5-HT<sub>7R</sub> agonist, LP-211, we demonstrate that 5-HT<sub>7Rs</sub> are critically implicated in synaptic plasticity of glutamatergic transmission in Purkinje cells, thus providing a novel cellular and molecular basis for the action of 5-HT at cerebellar level. In particular, we show that a brief application of LP-211 induces LTD at PF–PC synapse through the activation of postsynaptic 5-HT<sub>7Rs</sub>. Such depression was very similar in size, around 30–40%, to the long-term depression produced by stimulating parallel fiber axons in conjunction with depolarization of the Purkinje cell (Inoue et al., 1998 and Wang et al., 2000). Moreover, we show that 5-HT<sub>7R</sub> exerts a fine regulation of bidirectional synaptic plasticity by favoring the emergence of LTD vs. LTP. Treatment with a 5-HT<sub>7R</sub> antagonist altered the expression of PF-LTD, produced by pairing PF stimulation with PC depolarization; on the other hand, application of a 5-HT<sub>7R</sub> agonist impaired LTP induced by 1 Hz stimulation of PFs. The suppressive effect of LP-211 on LTP induction at PF–PC may provide a useful mechanism for preventing the simultaneous occurrence of conflicting forms of plasticity, such as potentiation of synaptic transmission, under conditions that promote postsynaptic LTD. Synaptic plasticity is considered a key mechanism for cerebellar signal processing and memory (Ito, 2001). For example, LTD at PF–PC synapses has been proposed to be the neural substrate for eye-blink conditioning and adaptation of the VOR (Ito, 1982, McCormick and Thompson, 1984 and Boyden et al., 2004); whereas LTP at PF–PC synapses takes part in the mechanisms underlying aversive associative memories such as fear conditioning (Sacchetti et al., 2004).

While 5-HT-LTD has already been described to occur in the nucleus accumbens, habenula and striatum (Mathur et al., 2011, Hwang and Chung, 2014 and Burattini et al., 2014), our data are the first, to the best of our knowledge, to show 5-HT-induced LTD at an excitatory synapse in cerebellar cortex of adult animals. It is likely that the 5-HT-induced depression at PF–PC synapses is mostly mediated by the 5-HT<sub>7R</sub> since it is strongly reduced by a selective 5-HT<sub>7R</sub> antagonist. Concerning the cerebellum, it has been reported that application of 5-HT facilitates the GABAergic transmission between cerebellar interneurons (i.e. basket, stellate and Lugaro cells) and PCs (Mitoma and Konishi, 1999 and Dieudonné, 2001). Thus, the overall action of 5-HT is to depress the activity of PC via suppression of excitatory inputs from PFs and facilitation of inhibitory inputs from interneurons. In such a way, 5-HT can potentially decrease the inhibitory drive of PCs to deep cerebellar nuclei neurons and ultimately refine the motor output. This hypothesis is in line with

previous findings showing a correlation between 5-HT level in the cerebellar cortex and motor activity (Mendlin et al., 1996).

#### **4.1. Mechanism**

5-HT<sub>7</sub>R-LTD engages a postsynaptic signaling pathway involving PKC and MAPK. This result is supported by previous studies in astrocytoma cells showing that serotonin via 5-HT<sub>7</sub> receptors can indeed activate p38 MAPK as well as PKC (Lieb et al., 2005). The ability of 5-HT<sub>7</sub>R to activate the ERK-MAPK pathway has also been demonstrated in cultured neurons expressing endogenous 5-HT<sub>7</sub>R as well as in cells transiently transfected with 5-HT<sub>7</sub>R (Errico et al., 2001, Speranza et al., 2013 and Speranza et al., 2015). The mechanism by which the 5-HT<sub>7</sub> receptors activate ERK1/2 has not been completely elucidated. According to some authors, activation of ERK1/2 is likely to occur as a consequence of the increase in both cAMP and calcium following activation of Gs-coupled 5-HT<sub>7</sub>Rs (Baker et al., 1998). Indeed, treatment with inhibitors of either calcium or adenylyl cyclase interferes with the 5-HT<sub>7</sub>R-mediated ERK activation (Guseva et al., 2014). Interestingly, activation of PKC by second messengers, calcium and diacylglycerol, has been shown to play an important role in the induction of cerebellar LTD (Tanaka and Augustine, 2008). For example, studies using PKC transgenic mice showed that chronic PKC inhibition restricted to cerebellar PF-PC synapses resulted in nearly complete suppression of both cerebellar LTD and adaptation of the VOR (De Zeeuw et al., 1998). PKC is responsible for the phosphorylation and consequent internalization of the AMPA receptors, which results in a decrease of postsynaptic density in PCs (Wang and Linden, 2000 and Tanaka et al., 2007). By recruiting Raf kinase, PKC can also induce the sequential activation of MEK, ERK1/2 and phospholipase A2 (PLA2; Yamamoto et al., 2012); PLA2 in turn produces arachidonic acid that binds and activates PKC. Once this loop is triggered, it causes PKC activity to be self-sustained for the maintenance of LTD. Based on our results, it is therefore likely that the activation of PKC and MAPK may also play a crucial role in the establishment of 5-HT<sub>7</sub>-induced LTD by governing the AMPA receptor phosphorylation and trafficking.

#### **4.2. Functional implications**

Neuroanatomical studies revealed that cerebellum receives projections from limbic and autonomic regions involved in emotional processing such as hippocampus, amygdala and hypothalamus as well as from brainstem areas containing neurotransmitters involved in mood regulation, including 5-HT (Dennis and Schutter, 2013). These connections provide the neural substrates for the

cognitive and emotional functions of the cerebellum (Schmahmann and Sherman, 1998, Timmann and Daum, 2010 and Strata, 2015). Conditions that have an emotional tone, or that produce anxiety or pain, can activate the cerebellar vermis. On the other hand, abnormalities in cerebellar function and structure have been documented in patients with depression and schizophrenia (Konarski et al., 2004 and Schmahmann, 2010). A well-studied form of emotional behavior involving the cerebellum is fear conditioning whereby an organism learns to associate a neutral stimulus to a painful stimulus (Maschke et al., 2002). When compared to healthy controls, patients with medial cerebellar lesions failed to show conditioned bradycardia as emotional response following repeated presentation of a tone with a painful stimulation. Interestingly, there are indications that memory of fear is accompanied by LTP at PF–PC synapses, which is consistent with the view that Hebbian learning occurs in cerebellar cortex during fear conditioning (Sacchetti et al., 2004 and Zhu et al., 2007). Since emotionally arousing experiences are associated with the release of 5-HT in various regions of the brain, including cerebellum (Storozheva and Proshin, 2011), we propose that the activation of 5-HT<sub>7</sub>R, by directly modulating learning-related synaptic plasticity in the cerebellar cortex, may affect not only the expression of conditioned fear, but also its consolidation and eventually extinction. The involvement of 5-HT<sub>7</sub>R in fear conditioning has been demonstrated in 5-HT<sub>7</sub>R KO mice, which displayed impaired contextual fear conditioning along with decreased long-term synaptic plasticity within the CA1 region of the hippocampus (Roberts et al., 2004). Based on preclinical findings, 5-HT<sub>7</sub>R are now emerging as a possible therapeutic target for the treatment of cognitive disorders and depression (Gasbarri et al., 2008, Matthys et al., 2011 and Volpicelli et al., 2014). For example, treatment with selective 5-HT<sub>7</sub>R antagonists has been shown to have anxiolytic and antidepressant effects in mice tested in the forced swim and tail suspension tests (Nikiforuk, 2015). In addition, some antipsychotics appear to interact directly with 5-HT<sub>7</sub>R (Andressen et al., 2015). Altogether these findings raise further questions: Is the cerebellar 5-HT<sub>7</sub>R signaling altered in these disease states? How and to what extent does the modulation of cerebellar 5-HT<sub>7</sub>R contribute to the therapeutic effects of numerous drugs interfering with 5-HT metabolism? Answering to these questions, prompted by our results on the role of 5-HT<sub>7</sub>R in cerebellar synaptic transmission and plasticity, may therefore provide new insights regarding the role of serotonin in cognitive processes and neuropsychiatric disorders involving the cerebellum.

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